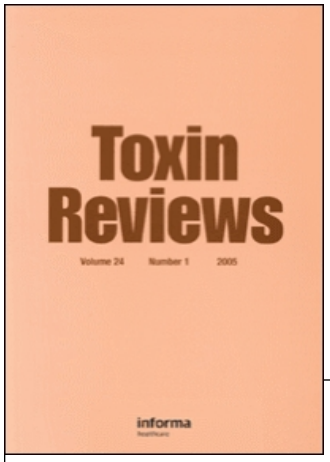

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Current Research on Reducing Pre- and Post-harvest Aflatoxin Contamination of U.S. Almond, Pistachio, and Walnut

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Current Research on Reducing Pre- and Post-harvest Aflatoxin Contamination of U.S. Almond, Pistachio, and Walnut

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ABSTRACT

Aflatoxins are considered to be potent carcinogens and teratogens to humans and farm animals. A variety of species of the fungal genus *Aspergillus* (mainly *A. flavus* and *A. parasiticus*) synthesize aflatoxins. Spores of these fungi are common in air and soil of agricultural areas of temperate and tropical environments. Because aflatoxigenic fungi are ubiquitous and opportunistic, aflatoxin contamination has become a food safety concern. The chief U.S. crops affected by the threat of contamination with aflatoxin include corn, peanuts, cottonseed, and certain tree nuts. Additionally, aflatoxin contamination has also become an international trade issue. Major trading partners of U.S. agricultural products have set total aflatoxin action threshold levels at four ng/g (ppb). This

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action level is far below the 20 ppb level recommended by the U.S. Food and Drug administration for domestic foods. Almonds, pistachios and walnuts are one of the major food commodities affected by food safety and trade issues associated with aflatoxin contamination. Commercial domestic production of these tree nuts in the U.S. is entirely in California. Moreover, 50 to 75% of domestically produced tree nuts are exported, chiefly to countries of the European Union (EU), which adhere to the four ppb action threshold level. Scientists at the USDA's Western Regional Research Center and the University of California, Davis' Department of Pomology and Kearney Agricultural Center have developed products and methods to reduce aflatoxin contamination of tree nuts. Control of insect pests in tree nut orchards is a major strategy to curtail aflatoxin contamination. Insect feeding damage can lead to fungal infection and concomitant aflatoxin contamination. This is especially the case with navel orangeworm on pistachio and almond. A new and potent lure has been developed to control codling moth, a major insect pest of walnuts whose feeding damage potentially leads to fungal infection. Through breeding and genetic engineering, new varieties of almonds and walnuts have been developed which are resistant to insect attack. New orchard management strategies have been prescribed to reduce reservoirs of *A. flavus* in tree nut orchards. A number of saprophytic yeasts, natural to tree nut orchards, have been discovered which show promise as biological control agents of *A. flavus*, in vitro, and are awaiting field testing. New and improved risk assessment models have been developed for sampling and measuring aflatoxin contamination through the processing stream and in bulk shipping lots of tree nuts. An automated sorter that detects and removes aflatoxin contaminated nuts from a processing stream in real time was developed. It was also concluded that methods currently used for hand-cracking of closed shell pistachios result in a higher risk of aflatoxin contamination. Perhaps the foremost breakthrough to date, however, is that constituents of walnut seed coat, especially from the cultivar 'Tulare', are potent inhibitors of aflatoxin biosynthesis, capable of rendering aflatoxigenic *A. flavus* virtually atoxigenic.

Key Words: Aflatoxin; *Aspergillus*; Tree nuts; Almonds; Pistachios; Walnuts; Insects; Navel orangeworm; Codling moth; Peach twig bore; Phytochemicals; Sorting.

INTRODUCTION

Aflatoxins are secondary metabolites produced by various species of *Aspergillus*. *Aspergillus flavus* Link and *A. parasiticus* Speare are the most significant species from an agronomic and food safety perspective (Diener

et al., 1987; Lewis et al., 1994; Payne and Brown, 1998). Aflatoxin B_1 (AFB1) and related difuranocoumarins are a concern to public health as potential carcinogens to humans and their proven toxicity to animals (Aguilar et al., 1993; Fujimoto et al., 1994; Hosono et al., 1993). AFB1 is generally considered to be hepatotoxic and a potent human liver carcinogen. Its mechanism of genotoxicity results from liver cytochrome P450 epoxidation of AFB1 to AFB1 *exo*-8,9-epoxide (AFBO). This epoxide reacts with DNA at the guanyl N7 atom after intercalation, forming a genotoxic DNA adduct (Essigmann et al., 1977; Johnson and Guengerich, 1997; Lin et al., 1977). Therefore, consumption of agricultural products contaminated with aflatoxins could result in acute hepatotoxicity and theoretically lead to chronic hepatocellular carcinoma (HCC) and mutagenesis in humans, although Stoloff (1989) argues against this.

Additionally, aflatoxin M_1 (AFM1), a metabolite of AFB1 found in milk of dairy cattle or lactating mothers exposed to aflatoxin, is of concern because of potential hepatotoxic and immunotoxic effects in infants and children. Likelihood of hepatotoxicity and hepatocarcinogenicity is greatly increased in developing countries where hepatitis B and C viruses (HBV and HCV) are endemic (Barraud et al., 1999; Henry et al., 1999; Stuver, 1998). Incidence of HBV and HCV has been increasing in the US adding to the concerns of aflatoxins in the domestic food supply. In response to these concerns, in 1994 the U.S. Food and Drug Administration (FDA) set guideline threshold levels for total aflatoxins in foods for domestic consumption at 20 ng/g (ppb) (DA, 1994). However, the European Union (EU) and Japan have a higher concern over the issue of aflatoxin contamination. As such, these countries have set their threshold levels for imported commodities at least five times lower, at four ppb and below.

THE TREE NUT INDUSTRY

A number of agricultural commodities are affected by contamination with aflatoxins (Robens and Richard, 1992). The principal U.S. crops of concern include corn, peanuts, cottonseed, and relevant to this chapter, tree nuts. The primary commercial tree nut crops affected by the threat of aflatoxin contamination are almonds, *Prunus dulcis* (Mill.) D.A. Webb, walnuts, *Juglans regia* L., and pistachios, *Pistacia vera* L.

In practice, essentially the entire U.S. almond, pistachio and walnut crops are produced in California. Of this domestically produced crop, approximately 60% are exported to other nations. The total U.S. commercial value of the three tree nut crops has steadily increased over the last two decades and currently stands at an annual value of about $2 billion (harvested crop).

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California produces 75% of the world's almonds. Almost 400,000 metric tons were harvested in 2001, a value of close to $1 billion. Almonds are the number one horticultural export from the U.S., at close to $700 million in value in 2000, followed by wine. Spain is the world's second largest producer, with a harvest about five times less than California. The chief importers of almonds are countries of the EU, India and Japan. Domestic walnut production is also overwhelmingly performed in California. The annual harvested value of walnuts has steadily increased over the past decade and fluctuates at around $300 million per year. The U.S. produces over 30% of the world's walnuts. China is actually the top producer, but the U.S. is the top exporter of walnuts, exporting close to 60% of its domestic production. Again, countries of the EU and Japan are the main importers of U.S. walnuts, followed by Canada. Iran is the world's largest producer and exporter of pistachios. The value of the U.S. pistachio crop is around $250 million per year, with about 50% of the harvest exported overseas. The main importers of U.S. pistachios are Hong Kong, countries of the EU and Canada (NASS, 2001).

In addition to the actual value of harvested and processed-shelled nuts, tree nuts have a substantial mark-up value in being added to a variety of edible consumer products. In fact, almost 40% of tree nuts consumed domestically are from breakfast cereals. Other types of value-added products include marzipan and other types of nut pastes, ice creams, and candies and bakery products (NASS, 2001).

TRADE AND FOOD SAFETY ISSUES

Aflatoxin contamination of tree nuts has become a growing international food safety concern for over a two decade period (Anonymous, 1979, 1993; Buchanan et al., 1975; Fuller et al., 1977; Morton et al., 1979; Phillips et al., 1980). A repercussion of this increasing concern has become the arguably very low threshold levels required to comply with CODEX Alimentarius standards on imported tree nuts.

The low thresholds for aflatoxin contamination have significantly increased the probability for rejection of tree nut shipments by the major importing nations of the EU and Japan. The EU initially rejected shipments of Iranian pistachios in 1998 and almonds from the U.S. in 1999. Because of current high level concerns in the EU about aflatoxin, there has been a continued embargo placed on importation of pistachios from Iran. The embargo, while opening more pistachio exports from the U.S., has increased awareness of potential for contamination of other tree nuts. In 1999, almost 70 tons of U.S. almonds were rejected by the EU. These rejections have increased pressure to ensure U.S. shipments of tree nuts are below

mandated aflatoxin action levels. The total cost of tree nut sales lost to aflatoxin contamination averages around $50 million/year, but can be much higher in years of greater insect damage (Cardwell et al., 2001). The impact of the potential for aflatoxin contamination in almonds, pistachios and walnuts, as food safety and international trade issues, has created a heightened desire to develop methods and strategies for reducing aflatoxins in pre- and post-harvest tree nut products.

There is a possibility aflatoxin might be used for agroterrorism. Following the Persian Gulf War, the United Nations Special Commission discovered a number Iraqi missiles with payloads of aflatoxin. In view of the non-acute toxicity of aflatoxin to humans, it is difficult to surmise what tactical military advantage aflatoxin-bombardment of opposing forces might confer to a military campaign. Exposure to aflatoxin might increase incidence of human liver cancer, but years after exposure (Zilinskas, 1997). Alternate targets of these weapons may have been agricultural commodities, such as the pistachio industry of Iran, where contamination would render them unexportable.

MECHANISMS FOR AFLATOXIN CONTAMINATION OF TREE NUTS

Insect feeding-damage is a principal factor leading to preharvest fungal infection of nut kernels of almond and walnut, and subsequent aflatoxin contamination. It is assumed insect damage also contributes to aflatoxin contamination of walnut. Wounds to the protective layers surrounding nut kernels (hull, shell and seedcoat) provide avenues for infection by wind-borne spores of aflatoxigenic aspergilli (Doster and Michailides, 1995, 1999; Klonsky et al., 1990; Phillips et al., 1976; Schatzki and Ong, 2001). The principal insect pests of tree nuts are larvae of the navel orangeworm (NOW), *Amyelois transitella* Walker (Lepidoptera, Pyralidae), infesting kernels of almonds, walnuts and pistachios, the peach twig borer (PTB), *Anarsia lineatella* Zell. (Lepidoptera, Gelechiidae), infesting meristem leaf shoots, husks and kernels of almonds, and the codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera, Tortricidae), infesting husks and kernels of walnuts. Infestation of tree nuts by insects entails a sequence of insect behaviors (Curtis and Barnes, 1977; Kuenen and Barnes, 1981). NOW females lay eggs on ''mummy'' nuts (stick-tight nuts from the previous season) in the fall through early summer (Sibbett and Van Steenwyk, 1993). NOW females do not normally lay eggs on immature nuts of the current season crop until those nuts mature at hull-split in August through early October (Barnes, 1977). However, NOW females will lay eggs on a current season crop before hull-split if nuts are already damaged by feeding of other insects (e.g., CM in walnuts and PTB

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in almonds) (Andrews and Barnes, 1982; Connell et al., 1989; Sommer et al., 1986) or, in pistachios, if nuts have prematurely split-open; so-called early splits (ES). Aflatoxin contamination of split-hull pistachios, without evidence of insect presence, has been reported, however (Sommer et al., 1986).

Alternate routes of infection may occur during development of the nut kernel or through natural breaches which take place as the kernel matures. The stem-end of the developing pistachio fruit hardens at a later point in development than the remaining tissues (Michailides, 1989). While this tissue is still soft, the kernel is vulnerable to being pierced by sucking-insects possessing stylet-like mouthparts. These insects are mainly various heteropterans such as leaffooted and stink bugs, common to pistachio and almond orchards (Gradizel and Dandekar, 2001; Michailides, 1989). In addition to proteolytic and hydrolyzing enzymes in their saliva, the stylets of such insects can also contain different types of microorganisms, including fungal spores, that can be co-injected into plant tissues along with the saliva (Campbell and Nes, 1983). This route of fungal infection presents a problem because there are no telltale signs of damage to the nut externally, making it difficult to remove such nuts from the processing stream. Pistachio nuts damaged externally by NOW or other chewing insects and later infected by fungi generally show some form of discoloration around the suture of the split hull. In pistachio, discoloration of the suture may occur without insect damage. This type of discoloration is readily detectable and such nuts can be removed from the processing stream (Pearson, 1996). However, spores of a number of species of *Aspergillus*, including *A. flavus*, can be detected in the internal tissues of pistachio, almond and walnut which exhibit no exterior damage (Bayman et al., 2002a). Though such nuts may not be contaminated with aflatoxin, proper post-harvest handling and storage of such tree nuts is required to prevent further colonization of internal tissues.

A major reservoir of *Aspergillus* spores is in the orchard litter surrounding tree nuts, especially pistachios. *Aspergillus* was found to frequently infect and sporulate on fallen fruit and male flowers (pistachios) throughout the summer in commercial orchards. A number of aflatoxin producing strains of *A. flavus* and *A. parasiticus* can be found in such litter. While it is not known whether there is direct infection of arboreal fruits, the infected litter contributes to increasing the probability of wounded nuts being infected by fungal spores (Doster and Michailides, 1994a).

RESEARCH EFFORTS

The economic return to tree nut producers and processors is directly related to the quality of their product. Presence of aflatoxins disrupts efficient

marketing of tree nuts and results in extra costs passed to the consumer. In some instances, after costs of harvesting, processing and shipping have been incurred, the product may be rejected from domestic or foreign markets. Currently available methods of removing aflatoxins from tree nuts after contamination are impractical and expensive (Scott, 1998). Moreover, use of fungicides to control aflatoxigenic aspergilli can have a contradictory effect in that sublethal doses may actually induce aflatoxin production (D'Mello et al., 1998). There is a need to design new and environmentally safe methods of reducing infection of tree nuts by aflatoxigenic aspergilli and to inhibit aflatoxin biosynthesis. The main thrust of research to reduce aflatoxin contamination of tree nuts is being performed by two groups of collaborating scientists in California whose research is funded by the United States Department of Agriculture's (USDA) Agricultural Research Service (ARS). One group includes a team of scientists in the Plant Mycotoxin Research Unit, Western Regional Research Center, USDA, ARS, Albany, CA. The other group includes scientists at the University of California, Davis (UCD), in the Department of Pomology and at the Kearney Agriculture Center. Efforts by these scientists focus on insect control, fungal control, orchard management and post-harvest sampling, detection and removal of contaminated nuts. These teams of scientists include individuals with expertise in insect biology, ecology, microbiology, plant pathology, natural product chemistry, plant breeding, genetic engineering, risk assessment analysis and agricultural engineering.

Reducing Pre-harvest Contamination

Insect Control

Developing better methods of insect control in tree nut orchards is a growing concern because of increased resistance to pesticides (Blomefield, 1994; Knight et al., 1994; Sauphanor and Bouvier, 1995; Varela et al., 1993). Moreover, recent regulations by the Environmental Protection Agency (EPA) (August 1999) are phasing out use of specific organophosphorous pesticides. This regulation is in response to the Food Quality Protection Act mandating strict reductions in pesticide use. This act also mandates eventual ban of some pesticides used for control of tree nut pests in the Central Valley of California. In spite of insecticide usage, harvested nuts have an annual rejection rate of 4 to 12 percent owing to insect and associated mold damage (Bentley, 1993).

Research and development of new methods to curtail insect feeding damage to tree nuts have involved a variety of approaches. Semiochemicals, chemical cues insects use for communication and discerning their

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environment, are being used to disrupt insect migratory, reproductive and host-finding behaviors. Plant breeding is developing almonds with better shell integrity and an improved suture seal that prevent infestation of the nut kernel by insects. Genetic engineering has developed transgenic walnuts that manufacture the insect-specific CRYL1A(c) endotoxin of *Bacillus thuringiensis* (Dandekar et al., 1998; Leslie et al., 2001). Improved methods for orchard management have been developed to remove mummies (unharvested nuts that remain on trees) that act as overwintering reservoirs for insects and reduce early-split nuts in pistachios that are frequently infested by NOW (Doster et al., 2001).

Semiochemical-Based Insect Control

Many insect behaviors, including feeding, mating, egg-laying and dispersal, are mediated by semiochemicals (Bell and Cardé, 1984). Dependency of insects on semiochemicals provides a unique means of monitoring pest populations and disrupting their normal behaviors as a means of control. Implementing use of semiochemicals is increasingly relevant in view of the tree nut industry's environmental and food safety concerns over pesticides. One category of semiochemicals includes sex pheromones. While multicomponent sex pheromones for PTB (Millar and Rice, 1992) and CM (McDonough et al., 1995) have been identified, the identification of components of the pheromone of NOW are incomplete. Synthetic reproductions of these pheromones have been effective on a commercial level for monitoring populations, but their use as mating disruptants has been unreliable. There is potential to attain requisite effectiveness of mating disruption by combining host-plant volatiles (HPVs) with pheromones (Light et al., 1993). PTB and CM vastly prefer fruit-hosts to nuts. There has been some success at exploiting pome fruit and stone fruit HPVs in tree-nuts. Commercial mating disruption systems for both species have had limited success and must be augmented with insecticide sprays (Rice and Jones, 1989).

The main constituent of the sex pheromone of PTB was identified as (*Z*)-5-decen-1-yl acetate (Roelofs et al., 1975). However, there was little success in using this compound for mating disruption (Rice and Jones, 1989). It was later determined that PTB sex pheromone contained the (*Z*)-5-decen-1-yl acetate and a (*Z*)-5-decen-1-ol, where the acetate was represented by >80% relative to the alcohol (Millar and Rice, 1992). After some initial indications of success, this formulation did not function fully as a mating disruptant (Rice et al., 1992). Examination of the pheromone of both a wild strain and a laboratory strain of PTB revealed two main components, (*E*)-5-decenyl acetate and (*E*)-5-decen-1-ol. However, the ratios of these components varied between the two strains with the major component being the alcohol at 98% in

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the wild strain and 89% in the laboratory strain (Roitman, unpublished results). The much greater presence of the alcohol component is opposite to that reported previously by Millar and Rice (Millar and Rice, 1992) and may explain the failure of the currently used formulation.

Chemical cues governing insect host-finding and oviposition in differing tree nut conditions are largely unknown. A number of volatiles reported from almond (Buttery et al., 1980a,b; Phelan et al., 1991) and walnut (Binder et al., 1989; Buchbauer and Jirovetz, 1992; Buchbauer et al., 1993; Buttery et al., 1986; Clark and Nursten, 1976, 1977; Nahrstedt et al., 1981) were reported in the past, but none included all tree nut tissues. A single preliminary analysis of volatile constituents of larval frass of NOW has been published (Lieu et al., 1982). Also, these pest insects have host-plants other than tree nuts (e.g., CM on pome fruits) whose volatiles might be effective attractants in a tree nut orchard. CM is attracted to odor of apples (Wearing et al., 1973; Yan et al., 1999). One apple volatile, (*E*, *E*)-α-farnesene, was found to be an attractant to CM based exclusively on laboratory bioassays (Hern and Dorn, 1999) and also to CM larvae (Landolt et al., 2000). The instability and rapid chemical breakdown of (*E*, *E*)-α-farnesene limits its use for controlling CM (Cavill and Coggiola, 1971). Gas chromatographic–mass spectrometric (GC–MS) analyses of HPVs of walnut leaves (Buttery et al., 1986; Campbell et al., 1999), pear leaves (Miller et al., 1989; Scutareanu et al., 1997), apples (Takabayashi et al., 1991), walnut husks (Buttery et al., 2000), and unripe apple or pear fruits (Buttery et al., unpublished results) showed a preponderance of mono-, sesqui-, and oxygenated-terpenoid HPVs. By contrast, HPVs of ripe fruits of apple and pear are predominantly aliphatic esters, a few short chain-length aliphatic alcohols, and several sesquiterpenes (Buttery et al., unpublished results; Carle et al., 1987; Nijssen et al., 1996). CM prefer pome fruits over walnuts (Barnes, 1991). In view of this preference, an array of volatile blends and individual HPVs of pome fruits were tested for attractancy to CM in a walnut orchard environment (Light et al., 2001). A pear-derived volatile, ethyl (2*E*, 4*Z*)-2,4-decadienoate, was discovered that is CM-specific, stable, and attracts male CM equivalent to female pheromone. Moreover, this kairomone also attracts female CM, both virgin and mated. Lures attracting females are of particular interest because they can be exploited to control the egg-laying life stage.

Effective kairomones for female moths are rare. Male lures are generally common, based on sex pheromones. This attractant provides a biorational alternative to conventional insecticide applications, while simultaneously ensuring food safety and reducing negative impact to the environment. Potential novel uses of this CM attractant in integrated pest management (IPM) include: 1) monitoring female flight patterns for prudential scheduling of insecticide applications; 2) monitoring pest emergence in orchards

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undergoing sex pheromone-based mating disruption, where monitoring with pheromone traps is unfeasible; 3) assessing whether female moths have mated; and 4) direct control of CM by mass-trapping, disrupting mating and ovipositional behaviors, or as an attracticide, where the lure would be combined with a pesticide. Another attracticide under testing is use of trap-trees, where adults are attracted to baited walnut trees genetically-transformed with *Bt*-toxin (Dandekar et al., 1994, 1998). In view of its unique properties to control one of the major agricultural pests in the U.S., the pear kairomone has been granted a patent (Light and Henrick, 2001).

Egg traps are the only current means of monitoring NOW populations. Such monitoring is needed for timing application of insecticides (Rice et al., 1976). The bait in NOW egg traps is a crude almond press cake impregnated with almond oil (Van Steenwyk and Barnett, 1985). Effectiveness of these traps is variable because of the crude, unrefined nature of the bait (Picuric-Jovanovic and Milovanovic, 1993). Evidence suggests attractancy of the bait involves long-chain fatty acids, especially oleic and linoleic acids (Phelan et al., 1991). However, more precise analysis of chemical composition is needed to improve the bait as a monitoring lure, attracticide, or ovipositional disruptant. A single-component sex pheromone of NOW has been identified but is not effective as a mating disruptant. Additional minor components of the female pheromonal emission of NOW have been identified. However, a new two-component blend has had mixed results in mating disruption trials (Millar et al., 1997; Shorey et al., 1998).

Other Strategies for Insect Control

Other non-pesticidal approaches to controlling insect pests of tree nuts include increasing constitutive natural products that are deterrents to insect feeding or improving the integrity of the hull and shell surrounding the nut kernel. For example, almonds possess low levels of cyanogenic compounds that could deter feeding by NOW (Dicenta et al., 2002). One such cyanogenic compound, amygdalin, can produce small amounts of hydrogen cyanide upon hydrolysis. Many lepidopterous insects, such as NOW, possess gut β-glucosidases that can perform this hydrolysis (Ferreira et al., 1998). One approach now being undertaken is augmenting amygdalin levels in certain almond tissues (Gradziel, unpublished results).

Another strategy to reduce insect damage is to improve the integrity of the endocarp (shell) surrounding the nut kernel. California almonds typically possess a 'papery' shell compared to the relatively more ''peach pit'' type of shell of Asian and European varieties. The thinner shells of California varieties increase the probability of becoming damaged during mechanical harvesting or of penetration by chewing or sucking insects, especially along the suture seal. Such damage can lead to fungal infection of the kernel. The

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weakened suture area was found to be associated with the developing funiculus. This discovery now allows trait selection for breeding almonds that will be more resistant to shell split (Gradizel and Dandekar, 2001).

Tree Nut Pests and Aflatoxin Interactions

The role of insects in facilitating infection of tree nuts by aflatoxigenic *Aspergillus* is well documented for pistachios and almonds. An interesting observation, however, is that many tree nut pests feed and develop normally on tree nuts which are heavily infected with fungi. Since these insects survive well in a highly fungal and mycotoxin contaminated environment, understanding mechanisms for their survival might provide either biological or metabolic clues on detoxification or avoiding toxicosis by mycotoxins. Efforts have been made in the past to identify microbial agents or products that degrade or inhibit synthesis of AFB1 (D'Souza and Brackett, 1998; Hamid and Smith, 1987; Munimbazi and Bullerman, 1998). However, degradation products and/or products within the aflatoxin biosynthetic pathway which might accumulate in lieu of the final aflatoxin product are frequently overlooked. Some degradative or pre-aflatoxin products, such as sterigmatocystin, can also be cytotoxic or carcinogenic (Klier and Schimmer, 1999; Pavlovicova, 1998; Wang and Groopman, 1999).

Metabolism of aflatoxins is intimately linked with toxic and carcinogenic effects. Accordingly, interspecies variations in AFB1-induced carcinogenesis or mutagenesis appear to be reflected in differences in metabolism, particularly in terms of cytochrome P450 (Cyt P450) and glutathione S-transferase (GST) activities. Cyt P450 monooxygenases are microsomal, membrane bound enzymes located in the endoplasmic reticulum of eucaryotic cells. For example, in humans the family of CYP3 cytochromes P450 catalyze epoxidation reactions of the terminal furan ring of AFB1 to AFBO. AFBO is highly reactive epoxide and is responsible for nucleic acid alkylation (Essigmann et al., 1977; Guengrich et al., 1996). GST, on the other hand, efficiently conjugates tripeptide glutathione (GSH) with the lipophilic electrophile, AFBO (Raney et al., 1992). This conjugation reaction is believed to be the primary detoxification pathway of AFBO. The significance of the interplay of enzymatic activities and respective biotransformation products is demonstrated in mice. Mice are much less likely to develop hepatocarcinoma than rats when exposed to AFB1 because of higher rates of GST activity and conjugation of AFBO with GSH in mice than in rats (Eaton et al., 1988).

Though chronic and acute lethal, and mutagenic, effects of AFB1 are reported, little is known about actual metabolism of aflatoxin by insects and respective biotransformation products. Aflatoxins have insecticidal, larvicidal, chemosterilizing and genotoxic properties against many insect species (Gaston and Llewellyn, 1980; Lamb and Lilly, 1971; Layor et al., 1976;

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Moore et al., 1978; Shibahara et al., 1995). AFL was the major in vitro metabolite identified in twelve genetically distinct strains of *Drosophila melanogaster* Meigen (Foester and Wurgler, 1984). In this *Drosophila* study, the Hikone-R strain, a selected strain for insecticidal resistance produced mostly aflatoxicol (AFL) and small amounts of AFM1 and aflatoxin B_{2a} (AFB2a). The relative amounts of these metabolites varied significantly among the strains of *D. melanogaster* examined. AFB1 can induce recessive lethal mutations in *D. melanogaster* (Labrousse and Matie, 1996). This insect possesses a Cyt P450 (CYP6a2) homologous to human CYP3a (Feyereisen, 1999). AFM1 was found to be a DNA-damaging agent in certain flies, but with an activity approximately 3-fold lower than AFB1 (Shibahara et al., 1995). Several species of cockroaches are less sensitive to aflatoxins than other insects (Llewellyn et al., 1988; Sherertz et al., 1978). Since cockroaches have varied diets, it is possible they evolved either resistance to naturally occurring aflatoxins routinely present in decaying matter or a means of excreting or sequestering the toxin in an inactive form. Infection of the sugarcane mealybug, *Saccharicoccus sacchari*, by either *A. parasiticus* or *A. flavus* has no entomopathogenic effects from aflatoxins (Drummond and Pinnock, 1990).

With regard to insects infesting tree nuts, larvae of NOW often live in a microenvironment in contact with mycelia, hyphae, and spores of aflatoxigenic fungi (Doster and Michailides, 1994a,b, 1999). Despite this contact this insect pest continues to develop and complete its life cycle. Larvae of CM, however, while frequently inhabiting walnut kernels that are highly infected with various fungi, have a lower potential for exposure to aflatoxin because walnut kernels are relatively antiaflatoxigenic compared with other tree nuts (Mahoney et al., 2000). The biotransformation products produced by these insects when exposed to aflatoxin were examined and compared to that produced by mouse and chicken (Lee and Campbell, 2000). A field strain of NOW produced three AFB1 biotransformation products, chiefly AFL, and minor amounts of AFB2a and AFM1. With AFL as a substrate, NOW larvae produced AFB1 and aflatoxicol M_1 (AFLM1). A laboratory strain of CM larvae exposed to AFB1 showed no detectable levels of any AFB1 biotransformation products in comparison to a field strain that produced trace amounts of only AFL. Neither NOW nor CM produced AFBO, the principal carcinogenic metabolite of AFB1. In comparison, metabolism of AFB1 by chicken liver yielded mainly AFL, whereas mouse liver produced mostly AFM1 at a rate eight-fold greater than AFL. Mouse liver also produced AFBO.

The relatively high production of AFL by NOW compared to CM may reflect an adaptation to detoxify AFB1. NOW larvae frequently inhabit environments highly contaminated with fungi and, hence, aflatoxin. Only low

amounts, if any, of this mycotoxin occur in the chief CM hosts, walnuts and pome fruits. Lee and Campbell (2000) concluded NOW larvae do not possess particular P450s for epoxidation of AFB1. However, biotransformation of AFB1 to AFL by NOW is generated by a cytosolic NADPH-dependent reductase. This study also suggested AFB1 reductase activity found in NOW larvae may result from a novel enzyme in view of involvement of GSH as an electron donor for AFL formation. Absence of the mutagenic biotransformation product of AFB1 in these insects, as compared to its production in mammals and birds (Manning et al., 1990; Neal et al., 1981) may have some eco-evolutionary basis. Both CM and NOW are major pests of tree nuts. The kernels of these nuts, if damaged, are prone to infection by fungi. Thus, these insects evolved in an environment of intimate contact with fungi and potential exposure to mycotoxins during larval development. This interaction between nut kernel-inhabiting insects and fungi may have existed for tens if not hundreds of millions of years as opposed to more recent interactions between mammals and aflatoxins.

Fungal Control

Fungal Associations with Tree Nuts

The association of *A. flavus* infection and contamination of tree nuts with aflatoxin has been reported numerous times beginning in the 1970s (Emami et al., 1977; Fuller et al., 1977; Lillard et al., 1970; Mojtahedi et al., 1979; Phillips et al., 1976; Schade et al., 1975). Surveys of the fungal communities inhabiting tree nut orchards have also been undertaken for pistachio, in Turkey (Denizel et al., 1976; Heperkan et al., 1994) and California (Doster and Michailides, 1994a,b) and almond in California (Phillips et al., 1979; Purcell et al., 1980). A comprehensive survey of the fungal flora found in California almonds, pistachios and walnuts and figs, collected from orchards and purchased from supermarkets, was also performed (Bayman et al., 2002a,b). While these latter studies identified *A. alliaceus* as the chief fungal species responsible for ochratoxin contamination of figs (Bayman et al., 2002b), this fungus was also identified on tree nuts (Doster and Michailides, 1994b). Moreover, though two other aspergilli reported to produce ochratoxin, *A. ochraceus* and *A. melleus*, were identified on some tree nuts, none of the strains identified produced ochratoxin. This study also found that the different tree nuts maintained a different set of fungal species as microflora, both on the surface and in internal tissues. The fact that spores of *A. flavus* were found in internal tissues reinforces the need for awareness of proper post-harvest handling of tree nuts. Such spores could

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serve as an inoculum should a favorable environment for germination arise. A further implication from this study may provide some knowledge towards the biological control of *A. flavus* or aflatoxigenesis. Current effective efforts at the biological control of *A. flavus* involve use of atoxigenic strains as biocompetitors of toxigenic strains in cotton fields (Cotty, 1994). Bayman et al. (2002a) were able to identify a number of fungal associations native to the tree nut orchard which showed reduced *A. flavus* populations. The strategy of using microorganisms native to tree nut orchards as biological control agents has also resulted in identification of a number of saprophytic yeasts (Hua et al., 1999). Many of the identified yeasts, mainly in the genera *Pichia* and *Candida*, have no pathology associated with humans. One isolate reduced aflatoxin production 100-fold relative to controls in in vitro studies.

Constitutive Natural Products

Application of fungicides, to prevent growth of microorganisms, and chemical treatment, to destroy aflatoxins, must be considered as unacceptable approaches to ensuring that shipments of tree nuts are within tolerance levels. A more appropriate general strategy is therefore to investigate natural products within the crop which confer resistance to *Aspergillus* colonization and growth, and/or aflatoxin biosynthesis. Two classes of protective natural factors exist in nature: phytoalexins, inducible metabolites, formed after invasion de novo, e.g. by activation of latent enzyme systems; and phytoanticipins, constitutive metabolites, present in situ, either in the active form or easily generated from a precursor. Since phytoalexins are produced only in response to fungal attack, it is obvious that their presence would lag behind the infection and levels capable of suppressing aflatoxin would be difficult to regulate. In contrast, phytoanticipins are always present and such factors offer the potential for enhancement through breeding and selection of more resistant cultivars, or even genetic manipulation to introduce or enhance their levels. Once such compounds have been identified, it is only necessary to ensure that they are present in large enough quantities and in tissues from which fungal growth and aflatoxin deposition must be excluded.

As mentioned above, although tree nuts appear to be shielded against infection by a series of protective layers that provide either chemical and/or physical barriers to microorganisms, they may nevertheless be contaminated with aflatoxins. These barriers include the husk or hull, consisting of outer (epicarp) and inner (mesocarp) layers; the shell (endocarp); and the pellicle, which is a thin, paper-like tissue (seed coat) surrounding the kernel. While the shell provides a physical barrier, it is not entirely homogeneous and is capable of being penetrated by insects that may introduce fungal spores at the suture

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and the stem end where the structure is less dense. Protective functions in the softer tissues such as the husk, pellicle, and possibly the kernel itself, are more likely to be dependent upon the presence of natural constituents.

The triterpenoids, betulinic acid, oleanolic acid, and ursolic acid have been shown to occur in high concentrations in almond hulls (Takeoka et al., 2000) but preliminary tests failed to show any significant anti-aflatoxigenic activity. In addition, 3-prenyl-4-*O*-*β*-D-glucopyranosyloxy-4-hydroxybenzoic acid, together with the ubiquitous phytochemicals, catechin and protocatechuic acid, have been isolated (Sang et al., 2002a) but these compounds have not been tested. Anacardic acids, natural constituents of the hulls of pistachios, have been shown to be capable to some extent of suppressing the biosynthesis of aflatoxins by *A. flavus* under laboratory conditions (Molyneux et al., 2000). However, hulls of walnuts are most highly resistant to *A. flavus* growth in comparison with other tree nuts such as pistachios and almonds.

A series of naphthoquinones in walnuts have also been shown to be potent inhibitors of aflatoxin biosynthesis. It is well-established that *Juglans* species contain a series of structurally related naphthoquinones and that these compounds occur in particularly high concentrations in the fleshy husk surrounding the nut (Binder et al., 1989). Moreover, leaves of the pecan [*Carya illinoensis* (Wangenh) K. Koch], another member of the Juglandaceae but in a different subfamily from *Juglans*, contain the naphthoquinone, juglone, which inhibits mycelial growth of *Cladosporium caryigenum* (Ellis & Langl.) Gottwald (=*Fusicladium effusum* G. Winter), the causative agent of pecan scab (Hedin et al., 1980). A crude extract from green walnut hulls, and pure juglone, have been tested for their activity against a wide range of microorganisms, including a variety of bacteria, filamentous bacteria, algae and dermatophytes (Krajci and Lynch, 1977). Although juglone has been evaluated against a number of plant pathogens (Sokolov et al., 1972), and juglone and plumbagin have been shown to be fungitoxic at high concentrations to 24 different fungi, including *A. flavus* (Tripathi et al., 1980), the effect of juglone and related naphthoquinones on aflatoxigenesis has not been investigated. We have therefore studied the activity of a series of these compounds in order to establish whether or not they are factors in resistance of walnuts to contamination by aflatoxins and, if so, the structural features contributing to such activity.

The effect on fungal viability and aflatoxigenesis of the four major naphthoquinones present in walnut husks: 1,4-naphthoquinone; juglone (5-hydroxy-1,4-naphthoquinone); 2-methyl-1,4-naphthoquinone; and, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), was studied in vitro. The quinones delayed germination of the fungus and were capable of completely inhibiting growth at higher concentrations. 2-Methyl-1,4-naphthoquinone and plumbagin had similar activity and were much more effective than the other

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two quinones, with germination delayed to 40 hours at 20 ppm and no growth at 50 ppm, whereas a control sample with no quinones present germinated in 16 hours. The effect on aflatoxin levels was highly dependent on the concentration of individual naphthoquinones in the media. At higher concentrations, aflatoxin production was decreased or completely inhibited, but at lower concentrations there was a stimulatory effect on aflatoxin biosynthesis, with >3-fold increase at 20 ppm of 2-methyl-1,4-naphthoquinone. Structural features associated with decreased fungal viability and greatest effect on aflatoxigenesis were the presence of a 5-hydroxyl or 2-methyl substituent, but there was no significant additive effect when both of these substituents were present (Mahoney et al., 2000). Of particular interest is the influence of these compounds in enhancing aflatoxin production at lower concentrations while reducing it at higher concentrations. It can be hypothesized that the naphthoquinones have a regulatory effect on certain genes in the gene cluster responsible for aflatoxin biosynthesis. The molecular biology of aflatoxin biosynthesis has been investigated in detail and the genes controlling specific steps of the pathway have been identified (Minto and Townsend, 1997; Payne and Brown, 1998). It may be significant that the early stages of aflatoxin biosynthesis, proceeding from norsolorinic acid to versicolorin A, involve hydroxylated anthraquinones that have structural moieties common also to juglone and plumbagin. Because of this structural similarity, naphthoquinones and the anthraquinone precursors may similarly affect domains of regulatory receptors which can up-regulate or down-regulate aflatoxin biosynthesis. Alternatively, *aflR* encodes for a zinc-containing, DNA-binding protein, and it is possible that the naphthoquinones act as chelators of this metal ion through sequestration by the 5-hydroxyl group adjacent to the quinonoid keto group. In any event, the effect of juglone and other walnut naphthoquinones on specific genes involved in aflatoxin biosynthesis warrants further investigation.

In vitro laboratory experiments, using 5% ground kernels in agar, have shown a significant difference in the ability of almonds and walnuts to support aflatoxin production, with walnuts being much less susceptible to contamination. Only one variety of pistachio, 'Kerman', is in commercial production and this fell between walnuts and almonds in aflatoxin production. On average, walnuts produced 0–28 μg/plate, the pistachio produced 40 μg/plate, and almonds produced 20–192 μg/plate. Moreover, there are varietal differences within each crop, with 34 varieties and breeding lines of almonds having a 10-fold range in aflatoxin levels while 26 walnut cultivars exhibited a 1400-fold range (Mahoney et al., 2002). The 'Tulare' variety of walnut completely suppressed aflatoxin production. This is the first example of any known crop plant affected by issues of aflatoxin contamination with complete resistance. Several other commercial walnut varieties, including 'Vina', 'Howard', 'Eureka' and 'Payne', all produced <5 μg/plate aflatoxin, whereas

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'Chico' produced 27 μg/plate. Black walnut (*Juglans nigra*) was the least inhibitory, with an aflatoxin output of 44 μg/plate. These results indicate a heritable natural resistance to aflatoxigenesis in walnuts, and to a lesser extent in almonds. These findings further suggest that selections of breeding lines for this characteristic can be made.

The particularly potent inhibition of aflatoxin biogenesis by 'Tulare' walnut indicated that attention should be focused on this variety, in attempting to elucidate the nature of the resistance factor. Sampling of the kernels over their period of development from June to September established that they had little resistance when first formed, with aflatoxin at 94% of control in June, but this level declined rapidly to 13% in July and was only 0.6% at maturity in September. Additional studies showed that the kernel resistance was not affected by rootstock or growing location and was therefore a trait of the 'Tulare' cultivar. In order to determine whether the resistance factor was localized in the seed coat or in the kernel without seed coat these were physically separated and all of the activity was found to reside in the seed coat. At a level of 0.5% seed coat in agar, the aflatoxin produced amounted to only 2% of control, whereas the kernels without seed coat produced no inhibition and at levels above 1% incorporation in agar, the aflatoxin levels rapidly increased, attaining 410% of control at 4% incorporation. This is probably a consequence of an increased supply of lipid and carbohydrate nutrients to the fungus. It therefore appears that the resistance factors are entirely located in the seed coat (Mahoney et al., 2002). Extraction of seed coat material with a series of solvents of increasing polarity and incorporation of the residual tissue after extraction into agar showed that nonpolar solvents removed very little of the anti-aflatoxigenic activity but substantial amounts were extracted by polar solvents such as methanol and water. The active constituents are therefore probably polar compounds, possibly phenolic in nature (Mahoney et al., 2002).

In contrast to the situation with walnuts, bioassay-directed fractionation of pistachio and almond kernels for anti-aflatoxigenic activity has not yet been attempted. However, a number of compounds have been identified in almond kernels, primarily as a consequence of a search for healthful food constituents. These include a sphingolipid, sterols (β-sitosterol and daucosterol), and nucleosides (uridine and adenosine) (Sang et al., 2002b). It is doubtful whether any of these compounds would have activity against aflatoxin biosynthesis and a strategy is now underway to search for anti-aflatoxigenic constituents, patterned on the successful approach used with walnuts. Different varieties of almond kernels bred for different oleic and linoleic acid balance showed varying degrees of supporting aflatoxigensis when inoculated with a toxigenic strain of *A. flavus*. However, the level of aflatoxin production could not be correlated with oil content (Gradziel et al., 2000).

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Cultural Practices

Practices involved in the production and processing of tree nuts can have profound effects on levels of aflatoxin in the finished product. Natural dehiscence (shell splitting) is a desirable feature of pistachios since most of the crop is marketed in-shell and the separation enables the shell to be easily removed by the consumer. If they are to be marketed, the undehisced portion of the crop must be sorted out and the shells removed before marketing as kernels (Crane and Iwakiri, 1982). Closed-shell pistachios are generally reprocessed overseas by water-soaking and artificial opening using low cost labor (Schatzki and Pan, 1996). The potential therefore exists for any nuts sequestering aflatoxigenic *Aspergillus* spores or aflatoxins to contaminate the batch during the rehydration process. In order to investigate the conditions under which such contamination could occur a study was undertaken to assess the extent of fungal propagation during reprocessing and to attempt to define the point of entry of *A. flavus* giving rise to aflatoxins in closed-shell pistachios.

Inoculation of fresh, or dried and rehydrated, closed-shell pistachios at the stem end of the shell with spores of *Aspergillus flavus* resulted in aflatoxin contamination of the kernel after incubation. The proportion of contaminated nuts was 48% for the fresh pistachios and 35% for the dried pistachios with 18% and 4%, respectively, having kernels containing aflatoxin levels in excess of 90 μg/kernel, sufficient to contaminate a 10 lb. test lot at the 20 ppb guidance level. Closed-shell pistachios batch-rehydrated for 3 hrs in a bath inoculated with *A. flavus* spores showed aflatoxin levels in the kernels of 170 ppb after 2 days incubation and the extraordinarily high level of 87,500 ppb after 6 days (Mahoney and Molyneux, 1998). This demonstrates that the kernels of closed shell pistachios can become highly contaminated with aflatoxin, even though the shell would appear to provide a physical barrier to the fungus. It has been shown that the stem end of the fruit remains relatively soft later in the season compared to the rest of the shell. This area is vulnerable to being pierced by the stylet-like mouthparts of heteropteran insects, which feed preferentially at this site (Michailides, 1989). It therefore seems probable that any fungal attack by aflatoxigenic *Aspergillus* species would also be most likely to occur through penetration of the stem end of the shell. These results strongly indicate that the practice of rehydration prior to mechanical splitting should be avoided.

Irrigation practices can also have a profound effect on risks associated with aflatoxin contamination of pistachios. Deficit irrigation of pistachio trees early in the growing season can lead to a phenomenon known as ''early-split nuts'' (Doster et al., 2001). In such cases, the shell and hull split open prior to harvest. Such splitting exposes the pistachio kernel to infestation by insects,

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especially NOW, and infection by aflatoxigenic aspergilli. Typically, the rate of early splitting in commercial pistachio orchards in California averages around 2–3% in a growing season. Experimental procedures using differently sized microsprinklers determined that the level of deficit irrigation in April or May, a period contemporaneous with shell growth, influences the incidence of early splits. Deficit irrigation at later stages during nut development does not appear to affect incidence of early splits. Hence, growers need to be aware of providing sufficient irrigation to pistachio orchards during early spring (Doster et al., 2001).

Reducing Post-harvest Contamination

Sampling Theory

When nuts, or other granular materials, are sampled for contaminants or other chemical inclusions, samples of a pre-selected number of nuts are withdrawn. These samples are then homogenized in some way and the contaminant concentration C is established by chemical or physical analysis. If this experiment is repeated a number of times, using the same sample size, N nuts, each time, the results will not be identical in general, but will form a *sample distribution*, $P(N,C)$. P will depend strongly on N, particularly in its breadth (standard deviation, s). For most nut products and practical sample sizes s is often quite large, as large or larger then the mean m of all the samples. Since one commonly wants the mean of the lot from which the samples are chosen, and which is represented by the sample mean m, such large standard deviations pose a serious problem in testing.

In addition to the sample distribution, there exist a more fundamental one, the *lot distribution*, p, which describes the probability that a single nut or granule, chosen from the lot at random, contains a concentration c of contaminant. This probability will generally depend on c, i.e. there may be more nuts at one concentration than another. We write p as $p(c)$. The lot distribution $p(c)$ is, of course, simply the sample distribution $P(1, C)$ when the sample size is 1. However, sample sizes of 1 are not practical. In typical nut lots the chance of any single nut being contaminated is exceedingly small (of the order of 1 in 10,000 to 100,000 nuts) and thus it would take an extraordinary number of samples and analyses to obtain any positive results. What is needed is to relate sample distributions and lot distributions directly, so that one can be derived from the other. In what follows, we shall use capitals when referring to samples values (which will depend on N) and lower case when referring to lot values.

To understand the relation between $p(c)$ and $P(N,C)$ the following analogy might be helpful. Imagine a barrel of black beans among which there are a small number of beans of differing colors, say white, red, blue, etc.

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Colors will be indexed by a subscript, i. These colored beans are assumed well mixed-in, but to be present in differing amounts, p_i. (Strictly, p_i is the fraction of all beans of color i.) With each colored bean there is associated a value, depending on its color: nothing for black ones, but with widely different amounts for the colored ones, say \$1 for white ones and \$1,000,000 for the most expensive color. We are, however, totally color blind: all beans look exactly the same to us. When we remove a sample of a fixed size, N, from the barrel, we can measure the total value, C, of the sample, but we cannot establish how many beans are colored or of which color they are. But what we can do is calculate the *probability* that the sample we have chosen will have the value C, as long as we know the p_i for each color. This is so because each color will form its own sample probability distribution, independently of the other colors. This distribution is the Poisson distribution that depends solely on Np_i. It tells us the chance that a sample of size N will contain exactly none, or one, or two, etc. beans of color i [The exact expression is that the probability of drawing a sample of k beans of color i is given by $P_k = \exp(-Np_i)(Np_i)^k/k!$]. If $Np_i \ll 1$, this probability drops off rapidly with k, so that the probability of having no contaminated beans in the sample is approximately unity, of having a single bean by Np_i, while the chance of finding more than one may be ignored. This situation remains if there are beans of many colors, but of varying concentration given by p_j, p_n, etc. Each forms its own Poisson and the probability of having several beans of different colors present is simply the product of the appropriate terms in the Poissons. As before, if $Np_i \ll 1$ for all i, only combinations containing a single colored bean of whatever color need be considered. To apply the situation to tree nuts, we replace the beans with nut kernels, the colors with concentrations.

To estimate a sample distribution from a lot distribution we chose N and pick at random an N-size collection of kernels of all contamination levels (including uncontaminated ones), each according to its own probability $p(c_i) = p_i$. For this we calculate the concentration of the sample C by summing over all the kernels. We then repeat this process a large number of times to obtain an estimate of the frequency for which the random N-sample falls into a set of limited ranges of C, which we designate C_i. This frequency is precisely the sample distribution $P(N,C_i)$. This method is called the Monte Carlo method and is commonly carried out by computer. The restriction $Np_i \ll 1$ for all i is not required here, but it does speed up the calculation.

To do the reverse, to estimate the lot distribution from the sample distribution is, in general, much more difficult. One approach has been taken by Whitaker and coworkers (Whitaker et al., 1972, 1974, 1994). They assume a parametric form for the lot distribution and use it to compute sample distributions in the manner discussed above. The calculated distributions depend on the parameters, of course. They then measure sample distributions

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by measuring many samples of the same size and, using standard statistical methods, compare these with their calculated ones. From ''best fit'' they obtain an estimate of the best parameters and thus the best lot distribution. This approach has a couple of disadvantages. First, the tests are rather insensitive to the parameters, so that one obtains rather poor estimates. Along with this comes the fact that certain resulting values, in particular the estimated mean concentration m, is particularly sensitive to the actual lot distribution, especially at high concentration. Again, such values are estimated poorly by these methods. Second, local manifestations of the lot distribution (location of maxima and minima, limits in the concentration and the like) can be very revealing of the processes causing the contamination in the first place. Such aspects are generally not part of any functional form of a previously studied distribution and are missed entirely if one uses a standard parametric form. For these reasons it is much preferable to use a method which is totally empirical and which can adapt to any shape of lot distribution.

On the other hand, if the restriction $Np_i \ll 1$ for all i is maintained, this approach allows the evaluation of the lot distribution from the sample distribution in straightforward manner (Schatzki, 1995a). To do so one estimates the sample distribution for an appropriate N by making several hundreds of sample measurements. [The value of N is chosen so that $Np(c_i)<0.1$, but not much less. This assures that the probability of obtaining a sample with 2 contaminated nuts is less than 5% of that of a single nut, but that the chance of getting at least some contamination in the range of C of interest is not much less than 10%. The appropriate value of N is chosen on the basis of a few trial experiments.] Since one now has at most a single significantly contaminated nut per sample at concentration c_i, one knows that the frequency of contaminated samples at concentration C_i is given by $P(N,C_i)=Np_i$, on the basis of the Poisson distribution, while the sample concentration C_i is given by $C_i=c_i/N$, by dilution. Thus p_i and c_i, which constitute the distribution of aflatoxin among single nuts in the lot, can be computed from the sample distribution and N. Interestingly enough, $P(N,C_i)$ on a log P vs. log C plot is just the lot distribution $p(c_i)$, shifted by log N in both axes. [The index i indicates binning in the log c axis, typically of half-decade width.] Any lot distribution, regardless of functional form or parameters, may be estimated. To cover the usual range of interest, say 10^2-10^6 ng/g, a few hundred measurements will suffice, rather than the few hundred thousand which would have been needed had one measured $P(1,C_i)$ instead.

Sampling Applications, Results, and Use

The above methods have been applied to lots of various types of nuts susceptible to aflatoxin contamination. Among US grown nuts these are

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mainly pistachios, almonds, occasionally walnuts and peanuts. The most common foreign grown nuts include Brazil nuts. Lot distributions have been calculated from sample distributions reported by others which the number of samples and the sample size N and were adequate to allow reliable estimates of the sample probabilities $P(N,C_i)$ and conversion to lot distributions ($P(N,C_i)<0.1$). In all cases $P(N,C_i)$ is expressed as the probability of a sample falling into a log C interval one half decade in size. Additional results were measured in our laboratory for other lots of interest. Typical results are shown in Figures 1, (Schatzki, 1995b, 1998; Schatzki and Pan, 1997) 2, (Schatzki and Ong, 2000, 2001) and 3 (Schatzki and Ong, unpublished results). Each curve represents 200–400 samples in our work, up to 700 samples in work of others.

The figures in most cases show lot distributions for lots expected to have high aflatoxin contamination and are not representative of high quality product in commerce. The latter might show levels 10 times or more lower.

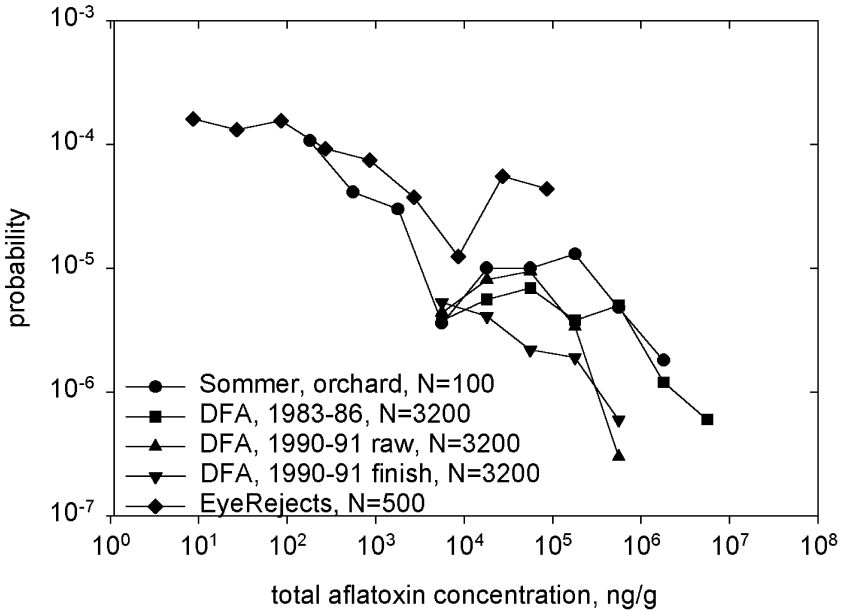

Figure 1. Total aflatoxin lot distributions computed from assorted pistachio sample distributions (see Schatzki, 1998). Aflatoxin lot distribution is the probability of a single kernel in a lot having aflatoxin content in a 3.16-fold range of aflatoxin concentration. Sample distribution is the probability of a sample from a group of samples of fixed size falling in such a range. Sample distribution will depend on sample size.

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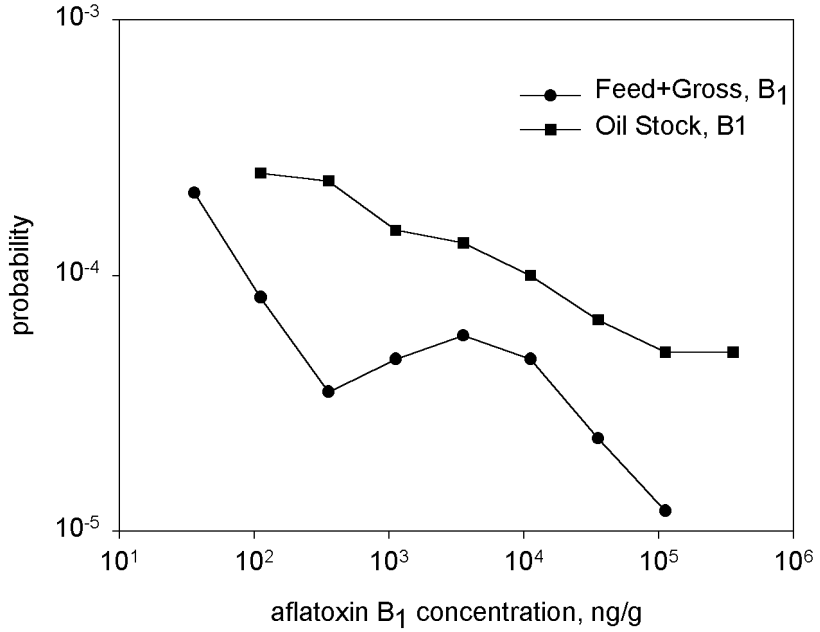

Figure 2. Aflatoxin B_1 lot distributions of insect damaged almonds (see Schatzki and Ong, 2001). Feed=insect damage indicated as feeding damage (removed kernel meat, not tunnels). Gross=insect damage indicated by presence of insect fragments or other filth. Oil stock=severely damaged almonds, based largely on insect damage.

Distributions will differ, depending on production and processing history and, of course, commodity. By and large, the distributions have been found to be rather flat from around 1000 ng/g to an upper limit, around 10^5-10^6 ng/g,. At this point they suddenly seem to fall to zero (no samples occur above the limit, suggesting that there is a limit to the nutrient supply for the fungus). Note that this uniform limit applies only to lot distributions, for samples distributions the limit appears at $10^5-10^6/N$ ng/g, since each positive sample contains but one ''hot'' nut. For $c<1000$ ng/g the distribution rises rapidly. They can not be tracked beyond $P(N,C_i)=0.1$ without a change of N. We have generally not done so, as it increases the work substantially. There is little interest in that part of the distribution function since the lot mean m is given by $\%_i\, p(c_i)c_i$ and thus dominates by the high end of the distribution. (Conversely, a much larger N might elucidate data above 10^5-10^6 ng/g.)

When lots of similar provenance, but differing processing severity or production constraints are compared one finds lot distributions differing in height, but have similar shape. An example is seen in Figure 3 for three peanut

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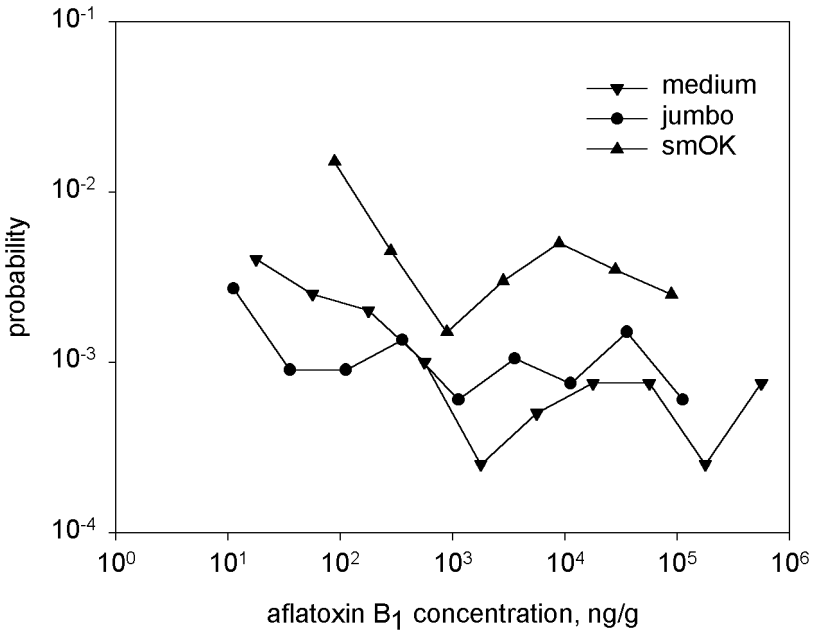

Figure 3. Aflatoxin B_1 lot distributions for lots of dry-farmed Florunner peanuts of various nut sizes (see Schatzki and Ong, unpublished results). Jumbo and Medium. Peanut sizes large enough to be acceptable for human consumption (>0.95 cm). SmOK. Peanuts falling through a 0.56cm screen.

sublots of the same dry-farmed Florunner peanut lot (Schatzki and Ong, 2000). The original lot was sorted for peanut size and those of the smallest size (smOK) show a lot distribution significantly higher but of similar shape than the sublots consisting of larger nuts. The medium size distribution lies slightly higher than that of the larger jumbos, but extends to higher concentration, which accounts in this case for its higher aflatoxin level. In peanuts it is well known that the smaller the nuts the higher the aflatoxin level tends to be. The smaller size sublots (such as smOK) are restricted from sale for human consumption, so in this case size sorting serves as aflatoxin sorting as well.

Once the lot distribution is known, the sample distribution may be computed for any sample size N. This may be made good use of when the sampling distribution is desired for a lot of very small p_i, when a large N is required. Such large N would require laborious and expensive sample measurements to construct a sample distribution directly. The difficulty can be avoided by obtaining the lot distribution, deriving the sampling distributions by random sampling by computer. The situation may be illustrated by an

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actual problem arising in connection with the testing protocol established by the EU for acceptance of pistachios (Schatzki and De Koe, 1999). This protocol (whose details will be avoided here for brevity) involves samples of 10 kg (N=approx. 9000), which must not exceed 2 ng/g of aflatoxin B_1 (4 ng/g total). Even such large samples are not adequate to represent a lot and the sampling distribution shows extensive variance. As a result, it is entirely possible that a sample, drawn from a lot having a mean m well below 2 ng/g, will still fail the acceptance test resulting in great cost to the shipper (costs exceeding $100,000/shipped lot). The shipper asks: ''What are the chances a lot, whose mean I know, will be rejected?'' or better yet ''How clean does my lot need to be so that I can expect a 95% acceptance rate?'' Similarly the buyer demands ''Given a lot passes acceptance, there should be at least a 95% probability that such a lot will pass possible subsequent tests, such as occur when a consumer group pulls retail samples.'' Such questions can be answered, using the calculations given here.

We proceed as follows. We measure the sample distribution of a lot of reasonably high infection with aflatoxin, but otherwise similar to the lot to be sold. This might be accomplished by a sample size of N=200 (adequate for $200p_i<0.1$) and perhaps 400 samples. From the resulting set of $p(c_i)$, we derive first the sample average concentration m as $\%_i\ p(c_i)c_i$. We next establish the sample distribution $P(9000,C_i)$ by Monte Carlo. We then compute the probability of acceptance from the integral$/_0^{2\ ng/g}\ P(9000,C_i)\ dC_i$, i.e., by simply establishing what fraction of the sample distribution fell below 2 ng/g. We will express that as $P(10\ kg<2\ ng/g|m)$, i.e. given m. We now multiply all the p_i by an arbitrary factor λ, (which represents the lot distribution at a different level of contamination) and repeat the entire process, obtaining $P(10\ kg<2\ ng/g|m)$, the probability of acceptance at this new contamination level. We keep changing λ, and so map out the probability of acceptance at all values of contamination of interest as a function of λm, [strictly, we map out $(P(10\ kg<2\ ng/g|\lambda m))^3$, because of details of the protocol] as we have done for the decreasing curve in Figure 4. As expected, the probability of acceptance drops as the lot average increases, reaching about 50% at m=2. Surprisingly, a lot must have an average aflatoxin level as low as 0.12 ng/g to be acceptable 95% of the time. American producers now produce a product at such levels for the European trade.

Lot distributions can also be made use of in designing sorting equipment. Since the lot mean m is dominated the $p(c_i)$ at high levels of c, knowledge of $p(c_i)$ in that region determines how much product needs to be discarded to reduce by a preset number. This is discussed in detail below.

Finally, the lot distribution curves can be used to directly guide cultural practices. Inspection of Figure 1 shows that the aflatoxin contamination is

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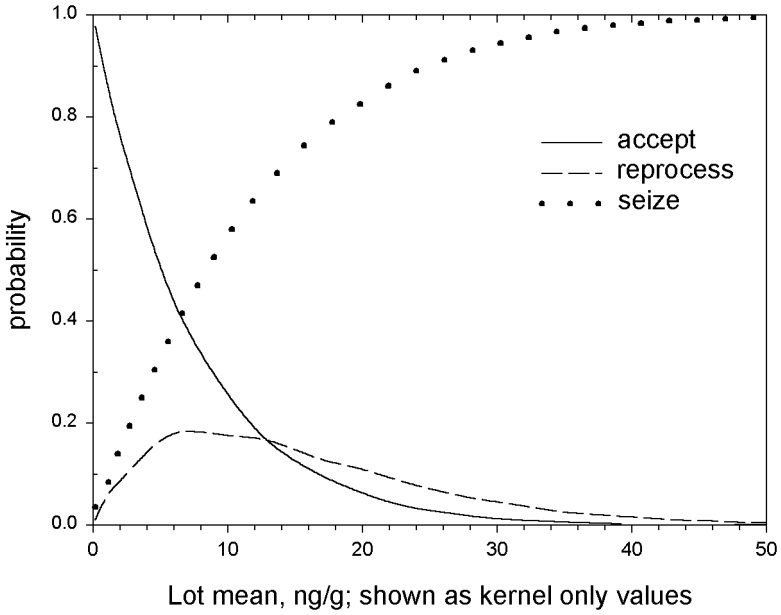

Figure 4. Probability of acceptance, required reprocessing and seizure for unshelled pistachios as a function of the lot mean aflatoxin concentration, using the EU acceptance levels (see Schatzki and De Koe, 1999). ''Accept''=acceptance of lot as is: each of three 10 kg samples tests as <2 ng/g total aflatoxin. ''Reprocess''=a resorting of the lot is allowed: a 30 kg sample tests as <5 ng/g total aflatoxin. ''Seize''=lot is confiscated: aflatoxin levels other than above.

not entirely independent of concentration for $c > 10^3$ ng/g, but shows a slight intermediate minimum, with similar shape for all curves. This shape may be directly associated with the onset of *A. flavus* contamination. It is only necessary to realize that production of aflatoxin occurs at an exponential rate as the mold grows and that henceforth the log c axis should be collinear with *time since fungal attack*. Furthermore, aflatoxin is only observed in pistachios for which the hull splits prior to harvest (which may vary from 1% to 8–10%, depending on the orchard) with additional contribution due to insect injury. Since early hull splitting commences about 6 weeks prior to harvest, comes to peak about 2 weeks later and ceases about two weeks prior to harvest, the lot distribution curve reflects this splitting, with the minimum corresponding to 6 weeks, the final drop-off to 2 weeks prior to harvest. The nuts splitting at the earliest time generate the most aflatoxin and good cultural practices suggest that insecticides should be applied at that time. The

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time constants derived from Figure 1 may be directly applied to Figure 2 where a similar shape curve is observed in insect damaged almonds. The derived times, here 4 weeks and immediately prior to harvest match the observed hull splitting in that commodity.

Sorting

Although aflatoxin content is of major concern in tree nuts, particularly in pistachios and almonds, little sorting to reduce this toxin occurs commercially. The reason is that extensive sorting already occurs for quality, resulting in a number of process streams and it is found that aflatoxin is associated only with a few low volume process streams. Removal of the pertinent streams, plus hand sorting in certain cases, can result in low aflatoxin counts.

In pistachios, major sorting steps are, in order, trash removal, water flotation to segregate empty-shell and immature nuts, hull removal, drying to 5–6% water content, sorting to remove closed-shell (again somewhat immature) nuts, electronic color sorting to segregate and remove stained shell nuts and, if required, hand sorting to complete the electronic process and also remove nuts with visible insect damage. Finally nuts are size sorted. In addition, closed-shell nuts are sent overseas for rehydration and manual cracking. However, this process is becoming outdated for US sales. Tests have shown (Schatzki and Pan, 1996) that high aflatoxin is found primarily in: 1) nut meats which have fallen freely from the shell; 2) very small nuts (>40 nuts/on.); 3) nuts showing insect damage and/or severe staining; 4) large ''floaters''; and 5) rehydrated nuts (presumably post-harvest, due to insufficient drying after cracking). Streams 1–4 are generally due to pre-harvest effects. Most of these substreams are of small enough volume that such nuts can be removed from commerce, either optionally or through marketing orders.

In the 1970s it was believed that the well known blue–green–yellow (BGY) fluorescence seen in aflatoxin contaminated corn could be used to detect aflatoxin in pistachios (Farsaie et al., 1978). On this basis Farsaie et al. (1981) developed a prototype sorter which performed well in removing such nuts. However, it was later realized that the BGY fluorescence indicated kojic acid, a co-metabolite of aflatoxin and that aflatoxin itself in tree nuts was of too low concentration to allow use of BGY for selection of aflatoxin contaminated nuts. A second approach to removing such nuts from process streams was based on the realization that toxin contamination was commonly higher in insect damaged nuts. Accordingly, considerable effort went into x-ray imaging of pistachios, (Keagy et al., 1996a,b) followed by the development of algorithms (Casasent et al., 2001; Sim et al., 1996) which could discern the presence of insect-caused holes in the image. The

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difficulty of obtaining x-ray images at a fast enough rate (single channel sorting rates of 40 nuts/s were required to match other commercial sorting equipment) and the emergence of better methods to detect nuts containing aflatoxin caused abandonment of this approach. The most successful method was based on the work of Sommer (Sommer et al., 1986) which indicated that only nuts in which the hull and shell split prior to harvest (ES) (about 2% of the total) showed aflatoxin. This level would increase if insect damage occurred as well (Doster and Michailides, 1994b; Sommer et al., 1986). Such splitting would allow access to the kernel and thus infection by *A. flavus,* required for aflatoxin to appear. This hull splitting resulted in a recognizable tannin stain of the shell which remained after process hulling and drying (Pearson et al., 1994). This stain pattern was utilized by Pearson who developed an image based sorter which could remove all aflatoxin contaminated nuts (up to 2% of total) at commercial rates and with acceptable false positive rejection (Pearson, 1996; Pearson and Schatzki, 1998). The sorter failed to perform satisfactorily during the occasional year when insect damage was so high that 2% removal was inadequate. So far, this sorter has not yet found commercial acceptance.

In the case of almonds, commercial sorting for quality results in rejects and a number of process streams of differing value. The higher value product consists of ''natural'' almonds, i.e. almonds still in the brown skin of the kernel (in-shell almonds have little market). Blanching results in lower value of ''manufacturing stock.'' Within each class increasing damage and/or cutting or grinding reduces value. Ostensibly, all nuts showing insect (similar bird or rodent) damage are rejected. A survey was carried out of results on all 1993 California crop material for which results and grade were recorded (Schatzki, 1996). It was found that aflatoxin was found essentially only on chopped or ground manufacturing stock, with concentrations increasing as the chop or grind became finer. This suggested that at least some lots with insect or similar damage (holes or broken surfaces) had been commutated to hide such damage and that aflatoxin occurred only following insect damage. Subsequent work on hand picked out insect damaged nuts indicated that only certain type of insect (gross and feeding) damage was associated with aflatoxin (Schatzki and Ong, 2001). Such damage is easily detected in natural almonds by color sorters. Thus, removal of this single stream should eliminate aflatoxin contamination in almonds. A method of detecting pinholes in almonds from x-ray imaging, similar to the work in pistachios, discussed above, was carried out as well (Kim and Schatzki, 2001), but in light of the pinhole results discussed above further research was abandoned. For walnuts, the main commercial sorting is carried out to separate the light colored (high value) shells from darker shells that develop during late harvest. The dark shells contain any shriveled or darkened

kernels. Work is currently in progress to relate such dark skin kernels to aflatoxin content.

SUMMARY AND CONCLUSION

The research efforts outlined above indicate the steady progress in producing tangible strategies and products for reducing aflatoxin contamination of tree nuts. New approaches to the control of insect pests of tree nuts are under development and some have already achieved success under field conditions. Additionally, a group of natural products has been identified from walnut that could render aflatoxin-producing aspergilli virtually atoxigenic. Mathematical models have been developed that assess the balancing act of the rigors for sampling for aflatoxin contamination juxtaposed to detecting contamination and amounts of shipped nuts required to be destroyed during sampling. Lastly, commercially viable sorters have been or are under development that will improve removal of contaminated products from the processing stream.

In conclusion, because one of the fundamental factors promoting preharvest contamination of tree nuts by aflatoxins is insect-feeding damage, identification of natural compounds present in tree nuts that deter insect feeding would be of additional value to the research efforts outlined above. Some volatile compounds affecting insect behavior may also have anti-fungal activity towards *Aspergillus* or be anti-aflatoxigenic. Thirdly, because aflatoxin genotoxicity results from its enzymatic transformation, mainly by a certain family of cytochromes P450, antioxidants may inhibit this process. Anti-insect, anti-fungal, anti-aflatoxigenic and anti-oxidant properties would directly lower contamination levels of aflatoxins in tree nut commodities, reduce risk to human consumers and lower chances of exported shipments being rejected. All of these properties exist in tree nut natural products. Identifying these natural products, such as the 'Tulare' walnut factor, could be followed by augmenting their amounts and optimizing respective bioactivities through modification of chemical structures. These procedures could be achieved using either conventional tree nut-breeding techniques or possibly through direct genetic engineering (Dandekar et al., 1994, 1998, 2002; Gradziel and Kester, 1994; Leslie et al., 1997; McGranahan et al., 1990). As outlined above, constitutive natural products can directly nullify aflatoxin biosynthesis; use of microbial-based antifungal natural products, such as iturins, are also promising (Moyne et al., 2001). Lastly, as more knowledge is gained concerning the molecular biology of aflatoxin biosynthesis interruption of the pathway, either genetically or with endogenous natural products, will become ever more achievable (Bhatnagar et al., 1998; Cary et al., 2000).

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